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# A Deletion Mutation in the SH2-N Domain of Shp-2 Severely Suppresses Hematopoietic Cell Development

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**Shp-1 and Shp-2 are cytoplasmic protein tyrosine phosphatases that contain two Src homology 2 (SH2) domains. A negative regulatory role of Shp-1 in hematopoiesis has been strongly implicated by the phenotype of *motheaten* mice with a mutation in the *Shp-1* locus, which is characterized by leukocyte hypersensitivity, deregulated mast cell function, and excessive erythropoiesis. A targeted deletion of 65 amino acids in the N-terminal SH2 (SH2-N) domain of Shp-2 leads to an embryonic lethality at midgestation in homozygous mutant mice. To further dissect the Shp-2 function in hematopoietic development, we have isolated homozygous Shp-2 mutant embryonic stem (ES) cells. Significantly reduced hematopoietic activity was observed when the mutant ES cells were allowed to differentiate into embryoid bodies (EBs), compared to the wild-type and heterozygous ES cells. Further analysis of ES cell differentiation in vitro showed that mutation in the *Shp-2* locus severely suppressed the development of primitive and definitive erythroid progenitors and completely blocked the production of progenitor cells for granulocytes-macrophages and mast cells. Reverse transcriptase PCR analysis of the mutant EBs revealed reduced expression of several specific marker genes that are induced during blood cell differentiation. Stem cell factor induction of mitogen-activated protein kinase activity was also blocked in Shp-2 mutant cells. Taken together, these results indicate that Shp-2 is an essential component and primarily plays a positive role in signaling pathways that mediate hematopoiesis in mammals. Furthermore, stimulation of its catalytic activity is not sufficient, while interaction via the SH2 domains with the targets or regulators is necessary for its biological functions in cells. The in vitro ES cell differentiation assay can be used as a biological tool in dissecting cytoplasmic signaling pathways.**

Recent advances in understanding the intracellular signaling system that mediates cell growth and differentiation have been facilitated by characterization of protein tyrosine phosphatases (PTPs) (7, 16, 54). The rapidly growing family of PTPs consists of transmembrane and intracellular forms that participate in multiple signaling pathways. PTPs, working together with protein tyrosine kinases (PTKs), control the overall protein phosphorylation levels on tyrosyl residues. However, PTPs do not simply play a negative regulatory role by reversing the phosphorylation effect of PTKs. Activation of a PTK in some cases requires a PTP activity. For example, dephosphorylation of $Tyr^{527}$ at the C-terminal tail of c-Src activates the kinase that in turn transduces a positive signal in cell proliferation (8).

The direct interplay between PTKs and PTPs in the control of cellular signaling has been strongly implicated by the finding of Src homology 2 (SH2)-containing PTPs, i.e., Shp-1 and Shp-2 in vertebrates and Csw in *Drosophila* species (1, 21, 38). Shp-1 appears to be predominantly expressed in hematopoietic cells, while Shp-2 is a ubiquitously expressed enzyme and closely related to *Drosophila* Csw (21, 38). The structure of this small group of PTPs immediately suggests a role in the regulation of proximal events downstream of growth factor receptors with intrinsic PTK activity. Indeed, the PTPs physically associate via the SH2 domains with ligand-activated growth factor receptors and other tyrosine-phosphorylated signaling proteins (20, 25, 29, 30, 57, 63). However, rather than observing a direct action on autophosphorylated receptor PTKs, many studies identified the PTPs as targets of various PTKs (21, 38). Shp-1 and Shp-2 can be rapidly phosphorylated on tyrosyl residues by activated receptor PTKs, which might alter the catalytic activity or regulate their interaction with other SH2-containing proteins. One well-described example is the detection of a physical complex of Grb2 and Shp-2 (6, 32, 57). It was proposed that Shp-2 might serve as an adaptor to recruit the Grb2-Sos complex to receptor PTKs, thereby linking to the Ras signaling pathway (6, 32). Challenge to this adaptor hypothesis has come from the observations that mutation of the putative Grb2-binding sites did not affect Shp-2 activity in mediating the growth factor stimulation of Ras and mitogen-activated protein (MAP) kinase (5, 55), while microinjection or expression of a catalytically inactive Shp-2 mutant suppressed the signaling pathway (5, 36, 39, 55, 72). It seems more likely that dephosphorylation of an unidentified substrate(s) by Shp-2 contributes to the Ras-MAP kinase activation. The physiological significance of tyrosine phosphorylation of the PTPs remains unclear, although the observations have been widely reported under various circumstances (21, 38). Recent identification of daughter of sevenless (DOS) as a possible downstream target of Csw (Shp-2) sheds light on the elucidation of its function in the Ras pathway (24, 46). DOS contains a pleckstrin homology (PH) domain at the N terminus and multiple presumptive tyrosine phosphorylation sites and functions

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between Sevenless and Ras1 in the control of *Drosophila* eye development.

The SH2 domains of the PTPs might also function to suppress the enzymatic activity of the phosphatase domain. Binding of the SH2 domains with a specific phosphopeptide significantly stimulates the PTP catalytic activity in vitro (27, 31, 41, 44, 53, 74). Likewise, truncated proteins of Shp-1 and Shp-2 lacking the SH2 domains exhibited enhanced phosphatase activity, compared to the wild-type full-length proteins in vitro (14, 40, 41, 59). Therefore, association of the PTPs via the SH2 domains with other molecules might not only control their access to regulators or substrates but also might simultaneously activate the PTPs. It should be noted, however, that the interaction of Shp-2 with the Jak-2 kinase does not involve the SH2 domains. Instead, a structural motif between the SH2 and the catalytic domains is required (75). Novel mechanisms might be involved in regulation of the interaction of Shp-2 with other proteins and the phosphatase function.

Mapping of the *Shp-1* gene to the *motheaten* locus has greatly expedited the elucidation of its functions in vivo. The mouse mutants *motheaten* and *viable motheaten* contain mutations in the coding region for the SH2 and the catalytic domains of Shp-1, respectively (28, 52, 62). The mutant mice suffer from immune deficiency and autoimmune diseases and exhibit numerous hematopoietic abnormalities, including augmented production and tissue accumulation of granulocytes and monocytes-macrophages, overexpansion of the $CD5^+$ B-cell subpopulation in peripheral blood, excessive erythropoiesis, and defective T-cell and NK cell functions (50, 61). These hematological disorders are apparently intrinsic to the hematopoietic progenitor cells, since these abnormal phenotypes can be transferred following transplantation of the mutant bone marrow cells into normal X-irradiated mice, and reconstitution of mutant mice with normal bone marrow rescues the hematopoietic system (50, 51). All of these findings point to a negative regulatory role of Shp-1 in the development and functions of hematopoietic and lymphoid cells. Consistently, Shp-1 has been found to attenuate signals emanating from receptors for interleukin 3 (IL-3), erythropoietin (Epo), granulocyte-macrophage colony-stimulating factor (GM-CSF), and CSF-1, and to mediate inhibitory signals transduced by immunoglobulin G Fc domains (FcγRIIB1), NK cell inhibitory receptor, and CD22 (10–13, 18, 43, 73).

Little is known about the biological functions of Shp-2 in mammals, since Shp-2 was originally cloned by either PCR or low-stringency hybridization approaches (21, 38). Genetic analysis of the *Drosophila* homolog of Shp-2, Csw, has indicated that this phosphatase functions as a positive signal transducer of Torso and Sevenless PTKs (2, 42). Csw participates in the Torso signaling pathway for the control of normal development of terminal structures during embryogenesis and is required for R7 photoreceptor cell differentiation by working together with DOS downstream of Sevenless. Overexpression of a catalytically inactive Shp-2 mutant interfered with the fibroblast growth factor-induced MAP kinase activation and the normal gastrulation process in *Xenopus* embryos (55). In order to determine the physiological functions of Shp-2 in the mammalian system, we have created a mutant *Shp-2* allele by deleting 65 amino acids in the SH2-N domain by homologous recombination in mouse embryonic stem (ES) cells. Homozygous mutant mice die around day 9.5 of gestation with severe defects in the organization of axial mesodermal structures and posterior development during gastrulation (48). Interestingly, the homozygous Shp-2 null mutant embryo dies at the same time (4), suggesting that the mutant Shp-2 protein with a deletion in the SH2-N domain is biologically inert. Early embryonic lethality has precluded further analysis of the function of Shp-2 in the differentiation of specific cell lineages. We have now isolated homozygous Shp-2 mutant ES cell lines and dissected the hematopoietic cell development in an in vitro differentiation system. We show here that differentiation of homozygous mutant ES cells into erythroid precursors is severely decreased and that differentiation into myeloid cell lineages is completely blocked. Reverse transcriptase (RT) PCR analysis of the mutant embryoid bodies (EBs) demonstrated deficient expression of several specific marker genes that are induced during early stages of hematopoietic cell development. This paper defines for the first time a cytoplasmic PTP as an essential effector in the development and regulation of the hematopoietic compartment in mammals.

## MATERIALS AND METHODS

**Cell lines and reagents.** Wild-type ES cells were grown in standard ES medium, Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, and 1,000 U of recombinant leukemia inhibitory factor (LIF) per ml (69). The targeting construct and selection strategy for a *Shp-2* mutant allele in mouse ES cells was previously described in detail (48). To isolate homozygous mutant ES cell lines, heterozygous ES cells were seeded on gelatinized plates at a density of 5,000 cells/ml and were selected in ES cell medium containing 2.0-mg/ml G418 according to a published method (37). Individual resistant clones were isolated and diagnosed by Southern blotting, RT-PCR, and immunoblot analyses. Epo, stem cell factor (SCF), IL-1α, and GM-CSF were kindly provided by Genetics Institute, Inc., and IL-3 was derived from WEHI-3 cell-conditioned medium, which was a gift from Scott Boswell, Indiana University.

**In vitro differentiation of ES cells.** Mouse ES cells were induced to differentiate in two different ways (65). In the one-step differentiation assay, single ES cells were seeded in methylcellulose-Iscove's modified Dulbecco's medium (IMDM) without LIF at a density of 2,000 cells/ml, and EBs with or without erythrocytes were counted at day 12. The number of EBs showing a distinctive red appearance was compared with the total number of EBs. In the two-step assay (26, 64), ES cells were first cultured as described above and the EBs were harvested at the indicated times. The EBs were gently dissociated by collagenase digestion and passage through a 21-gauge needle. Single cells were replated in the same medium supplemented with various growth factors. CFU were evaluated at day 7 of secondary plating for primitive and definitive erythroid colonies (CFU-$Ery^P$ and -$Ery^D$, respectively) and at day 12 for GM colonies and mixed erythroid-myeloid (CFU-Mix) and mast (CFU-Mast) cell colonies. The number of $Ery^P$ CFU (CFU-$Ery^P$) was counted with the addition of Epo; CFU-$Ery^D$ was counted with Epo plus SCF, CFU-GM and CFU-Mix were counted with Epo plus SCF plus IL-1α plus IL-3 plus GM-CSF, and CFU-Mast was counted with SCF plus IL-1α plus IL-3. The dosages of the growth factors used were as follows: Epo, 5 U/ml; SCF, 200 ng/ml; IL-1α, 1,000 U/ml; IL-3, 100 U/ml or 5% WEHI-3 cell conditional medium; and GM-CSF, 50 U/ml. Experiments were performed with three independent clones of homozygous mutant ES cells isolated from two independent heterozygous mutant ES cell lines. The heterozygous ES cell clones included in the experiments had undergone selection with high concentrations of G418.

**RT-PCR.** ES cells were seeded into semisolid methylcellulose IMDM for differentiation. Resultant EBs were harvested at days 0, 3, 6, 10, 14, 18, and 22, and total RNA was extracted by using the RNeasy Total RNA kit from QIAGEN. The RNA preparations were digested with DNase to eliminate any remaining genomic DNA. A random hexamer-primed cDNA strand was synthesized with mouse mammary leukemia virus RT. The RT products were used as templates for PCR amplification by using gene-specific primers (35, 64, 68). The amount of cDNA synthesized was calibrated by using the relative expression level of hypoxanthine phosphoribosyltransferase (HPRT) as a standard. Briefly, PCR was performed with primers specific for HPRT by using a series of dilutions of cDNA synthesized from known amounts of total RNA. The PCR amplification efficiency for each pair of primers was determined empirically by checking the amounts of the PCR product on agarose gels after various cycles to maintain amplification in a linear range. RT-PCR analysis then permitted comparison of the relative levels of specific RNAs among different samples.

**MAP kinase assay.** Cells were starved in serum-free ES medium plus 0.5% bovine serum albumin for 24 h when they reached about 80% confluence. Stimulation was performed with SCF (100 ng/ml) for different times at 37°C. Cells were then washed twice with cold phosphate-buffered saline and lysed in PLC-LB cell lysis buffer (22). The protein concentrations of the lysates were quantitated, and MAP kinase was immunoprecipitated from cell lysates of 300 μg of total proteins by using a polyclonal antibody against Erk1, together with protein A-sepharose (PAS) beads. The immunocomplex was washed twice with HNTG (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol plus 1 mM $Na_3VO_4$) and once with kinase buffer (20 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 1 mM $Na_3VO_4$). The reaction was carried out in a mixture containing 20 μl of kinase buffer, 1-mg/ml myelin basic

protein (MBP), 80 μM ATP, 0.075-μCi/μl [γ-$^{32}$P]ATP at 30°C for 10 min. The beads were spun down, and 18 μl of supernatant was spotted onto Whatman P81 paper. The papers were washed with 180 mM phosphoric acid and ethanol, air dried, and counted on a Beckman scintillation counter.

**Phosphatase assay.** The in vitro tyrosine phosphatase activity was measured as described previously (20). Briefly, bovine MBP was labelled with $^{32}$P upon phosphorylation by p60$^{c\text{-}Src}$ kinase (Oncogene Science). Shp-2 from wild-type and mutant cells was precipitated from 500 μg of cell lysates with anti-Shp-2 antibody (Santa Cruz) and mixed with the $^{32}$P-labelled MBP for different times, and the free $^{32}$P released into the supernatant was measured after trichloroacetic acid precipitation. The phosphatase activities of the wild-type and mutant proteins were evaluated by the extent of MBP dephosphorylation in vitro.

## RESULTS

**Isolation and characterization of homozygous Shp-2 mutant ES cell lines.** To assess the role of Shp-2 in differentiation of hematopoietic cells, we isolated homozygous Shp-2 mutant cell lines. In the targeted mutant ES cells, exon 3 in the *Shp-2* locus, encoding amino acids 46 to 110 in the N-terminal SH2 domain, was deleted by homologous recombination and was replaced by a neomycin resistance cassette (48). By selecting the heterozygous mutant cells in high concentrations of G418, we isolated seven homozygous mutant ES cell clones from two independent heterozygous ES cell lines. The genotypes of heterozygous and homozygous mutants were assigned by Southern blot analysis (Fig. 1A). RT-PCR analysis with primers flanking the deleted exon 3 region yielded a product of 366 bp from mutant cells, compared with a 561-bp fragment from wild-type cells. The mutant transcript was apparently produced by aberrant splicing to join exons 2 and 4, albeit with low efficiency (Fig. 1B) (46). Immunoblotting with antibodies against the C-terminal tail (residues 576 to 593) of Shp-2 detected a 57-kDa mutant protein (Fig. 1C), which was also observed in homozygous mutant embryos and fibroblast-like cells (48). The level of the mutant protein was approximately 25% of that of the wild-type protein.

**Reduced hematopoietic activity of Shp-2 mutant EBs.** ES cells derived from the inner cell mass of blastocysts are totipotent cells that undergo differentiation into various committed cell types, including blood cells in vitro (9, 17, 49, 65, 67). This system recapitulates hematopoietic development during the early stages of mouse embryogenesis (26, 65). In the present study, we assessed the effect of the Shp-2 mutation on hematopoietic cell differentiation of ES cells. Single ES cells were seeded into semisolid methylcellulose medium in the absence of LIF for differentiation into EBs that contain a variety of differentiated cells, particularly visible erythrocytes. We found that in contrast to the wild type, EBs of Shp-2 mutant origin contained few recognizable erythrocytes. Shown in Fig. 2A is a quantitative comparison of wild-type, heterozygous, and homozygous mutant ES cells. A significantly lower number of homozygous mutant EBs with erythrocytes compared to those for the wild type and heterozygotes was observed at day 10. Even in these positive mutant EBs, fewer erythrocytes were present, unlike the wild-type and heterozygous EBs that were surrounded with many visible blood cells. The total plating efficiencies were similar, with that of the homozygous mutant being slightly higher, which might be due to the decreased differentiation capacity of mutant cells (Fig. 2A). Shown in Fig. 2B are a typical wild-type EB surrounded by erythroid and myeloid cells and a representative homozygous mutant EB without visible blood cells. This result suggested defective hematopoietic development of ES cells mutant for Shp-2.

**Impaired development of erythroid cell lineages.** To further dissect the involvement of Shp-2 in differentiation of various

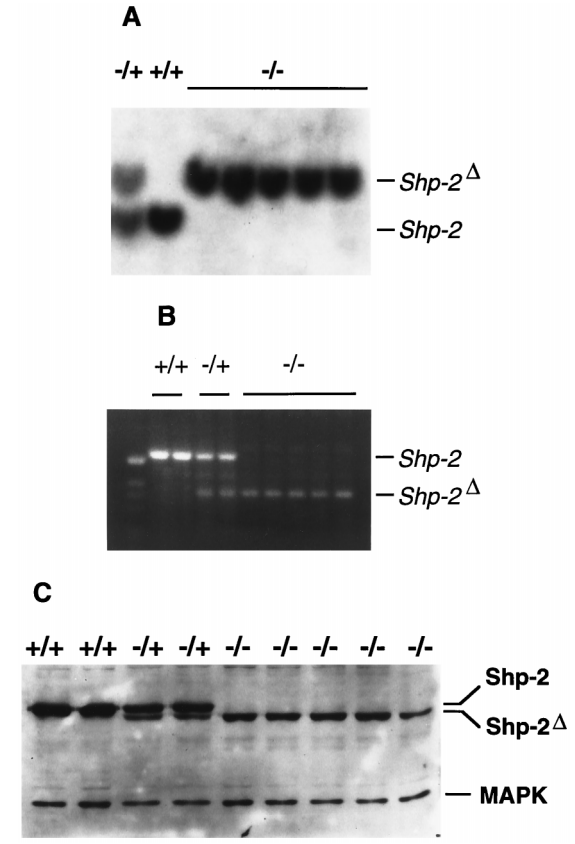


FIG. 1. Identification of targeted Shp-2 mutant ES cell clones. +/+, −/+, and −/−, wild-type, heterozygous, and homozygous mutant ES cells, respectively. (A) Southern blot analysis. Genomic DNA (30 μg) extracted from each cell line was digested with *Nco*I, resolved on a 0.6% agarose gel, and transferred to a Nylon membrane. Hybridization was performed with a $^{32}$P-labelled specific probe external to the targeting construct (48). (B) RT-PCR analysis. Total RNAs were extracted from the wild-type, heterozygous, and homozygous mutant cells, and RT-PCR was performed with the primers GGCAGAGAATCTCCTGCT and TACCCCAAGAAGAACATT, corresponding to amino acids 16 to 21 and 197 to 202 of Shp-2 (20), which flank the deleted amino acids 46 to 110. (C) Immunoblot analysis. ES cells were harvested in phosphate-buffered saline and lyophilized. Equivalent amounts of proteins (40 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and blotted onto a nitrocellulose membrane. Polyclonal anti-Shp-2 antibody against amino acid residues 576 to 593 in the C-terminal tail (Santa Cruz Biotechnology, Inc.) was used to detect Shp-2 expression, which was followed by a second antibody conjugated with horseradish peroxidase. The filters were developed by chemiluminescence reaction and exposed to radiographic film.

blood cell lineages, we set up a two-step in vitro ES cell differentiation assay (26). ES cells were first plated in semisolid medium and developed into EBs that contain hematopoietic cell precursors. The resultant EBs were dissociated at days 6, 10, and 14, and single EB cells were replated into semisolid medium supplemented with various cytokines for a CFU assay. It has been shown that the onset of hematopoiesis within EBs is not influenced by growth factors, while the survival and expansion of hematopoietic precursors developed in the EBs are improved by the addition of cytokines in the second step for CFU assay (26). Primitive erythrocyte colonies (CFU-Ery$^{P}$) were counted at day 7 after replating, with the addition of only Epo. In agreement with previous reports (26, 60, 64), the Epo-responding precursors gave rise to small colonies consisting of large nucleated cells (Fig. 3). Ery$^{P}$ was the first precursor to develop in EBs, and, as shown in Fig. 4, The highest efficiency of Ery$^{P}$ production was observed from day-6 EBs of

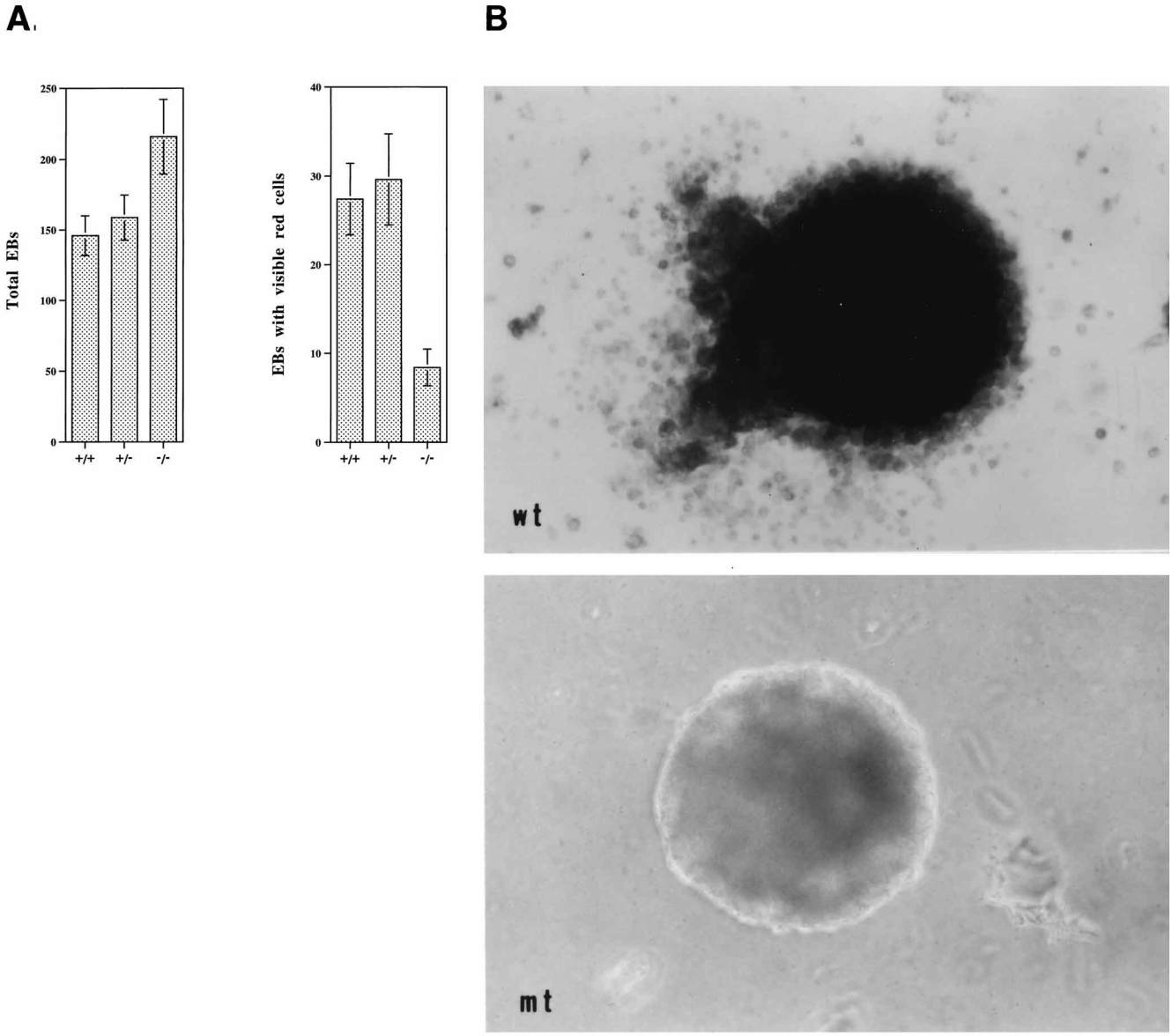


FIG. 2. Total and hematopoietic EBs on day 12 of differentiation. Wild-type (+/+), heterozygous (−/+), and homozygous (−/−) mutant ES cells were seeded at a density of $2 \times 10^3$ cells/ml in methylcellulose IMDM. (A) Total numbers of EBs and the numbers of EBs with visible red blood cells were counted. The data represent EB-forming efficiency of $3 \times 10^3$ cells and are average results from five plates. The experiments were repeated three times, and reproducible results were obtained. (B) A typical EB of wild-type origin surrounded with blood cells (wt), and a representative EB of homozygous mutant with no surrounding differentiated blood cells (mt).

wild-type origin, with a progressive decrease occurring by days 10 and 14, respectively. However, cells of day-6 EBs of homozygous mutant origin gave rise to only a few $Ery^P$ colonies (11% of wild-type levels), and those of later EBs gave to almost none. The numbers of $Ery^D$ colonies, which were assessed from growth in Epo plus SCF, were also drastically reduced from those for mutant ES cells (Fig. 4), suggesting that Shp-2 is involved in mediating the development of both $Ery^P$ and $Ery^D$ precursors.

**Block to myeloid cell differentiation.** By the two-step assay as described above, experiments were also performed to evaluate the effect of the *Shp-2* mutation on myeloid cell development. Numbers of CFU for GM, mixed erythroid and myeloid cells, and mast cells were counted on day 12 of secondary plating (Fig. 3 and 4). CFU-GMs were enumerated by replating EB cells in the presence of Epo, IL-1α, IL-3, SCF, and GM-CSF. As shown in Fig. 4, CFU-GMs were detectable for wild-type and heterozygous cells at days 6, 10, and 14, whereas no CFU-GMs were observed from the homozygous mutant EBs at any time examined. Similar results were obtained for CFU-Mix in the presence of Epo, SCF, IL-1α, IL-3, and GM-CSF. The kinetics of mast cell precursor development within EBs is slower than those of the other lineages (Fig. 4), in agreement with a previous report (26). No CFU of mast cells were observed in the presence of SCF, IL-1α, and IL-3 until day-10 EBs. Precursors for mast cells were absent from Shp-2 homozygous mutant EBs (Fig. 4). The impaired capacity of homozygous mutant ES cells for hematopoietic differentiation was observed for three independent clones, which were originally derived from two independent heterozygous clones.

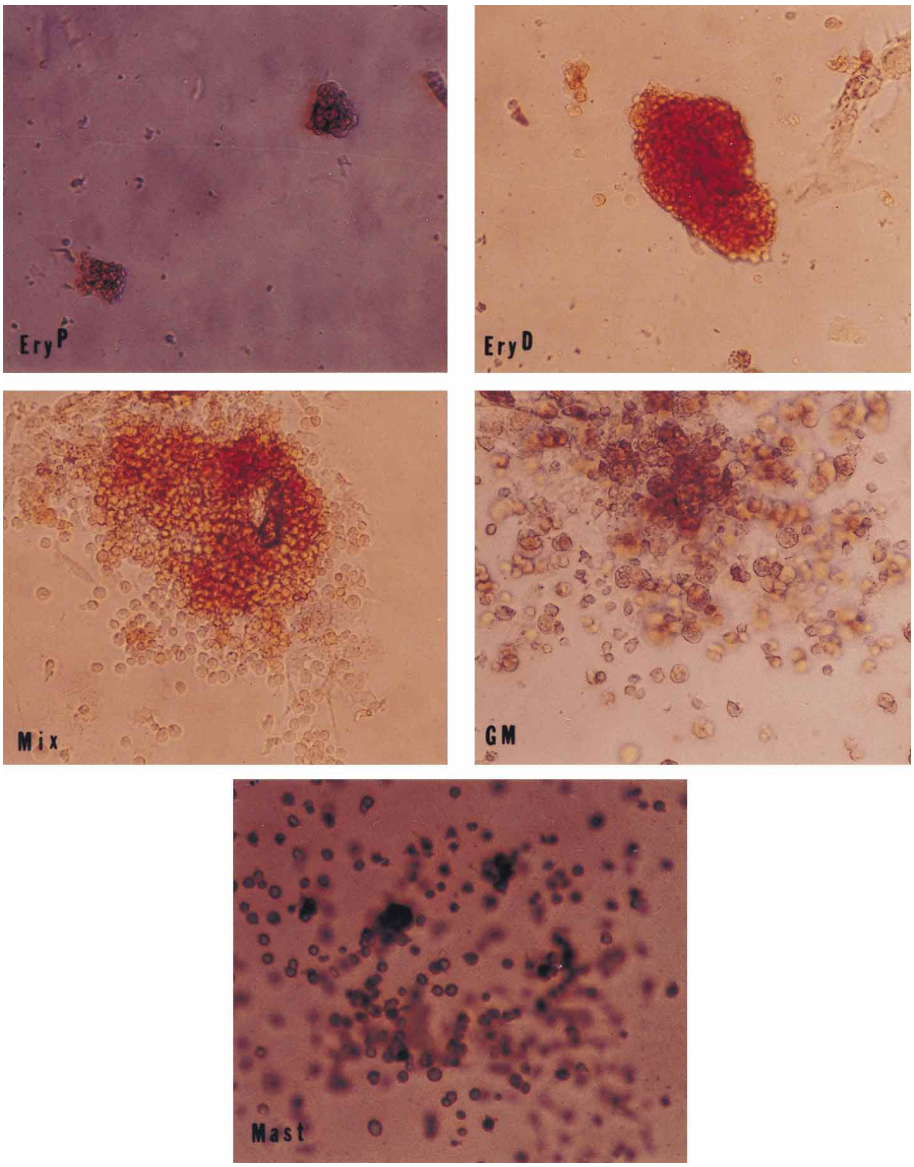


FIG. 3. Detection of specific cell lineages by CFU assaying. With the two-step ES cell differentiation system as described in the text in detail, generation of progenitors for each blood cell lineage was quantitated by counting individual colonies. Shown in this figure are representative colonies of Ery$^P$, Ery$^D$, GM, mixed erythroid-myeloid cells (Mix), and mast cells.

Thus, the mutation introduced into the *Shp-2* locus results in markedly reduced erythroid and myeloid lineage development.

**Reduced expression of blood cell marker genes.** The results described above point to the participation of Shp-2 in controlling the development of the myeloerythroid lineages. To corroborate the in vitro colony assays, we examined the expression of several genes that serve as markers of hematopoietic cells by RT-PCR analysis (35, 64, 68). Total RNAs of wild-type and mutant origins were isolated from ES cells and from EBs collected at selected days. As shown in Fig. 5A, transcripts of wild-type and mutant Shp-2 were detected at all times. RNAs encoding the hematopoietic transcription factors GATA-1 and PU.1 were reduced in abundance in EBs of mutant origin, in agreement with impaired myeloerythroid differentiation (Fig. 5B). In agreement with this, transcripts for the cytokine receptor components AIC-2B, c-fms, and IL-7R were also reduced. Moreover, a marked decrease in the levels of β-H1 and β-major globin transcripts correlates well with reduced formation of Ery$^P$ and Ery$^D$ colonies. Transcripts for the c-kit receptor, which is expressed in both hematopoietic and nonhematopoietic compartments, were equivalent between wild-type and mutant EBs. A modest reduction in the abundance of CD34 transcripts was observed for mutant EBs. These findings reflect a selective loss of expression of blood cell-specific markers, in agreement with the impairment of hematopoiesis demonstrated by in vitro differentiation colony assays.

**Phosphatase activity of the mutant Shp-2 in vitro.** The targeted mutation resulted in an in-frame deletion of 65 amino acids in the N-terminal SH2 domain of Shp-2 without interrupting the catalytic domain. Consequently, a mutant protein of Shp-2 with the internal deletion was expressed with less efficiency, 25% of that of the wild type. Since several groups observed an elevated level of phosphatase activity in vitro when the SH2 domains were deleted (14, 40, 41, 59), we tested

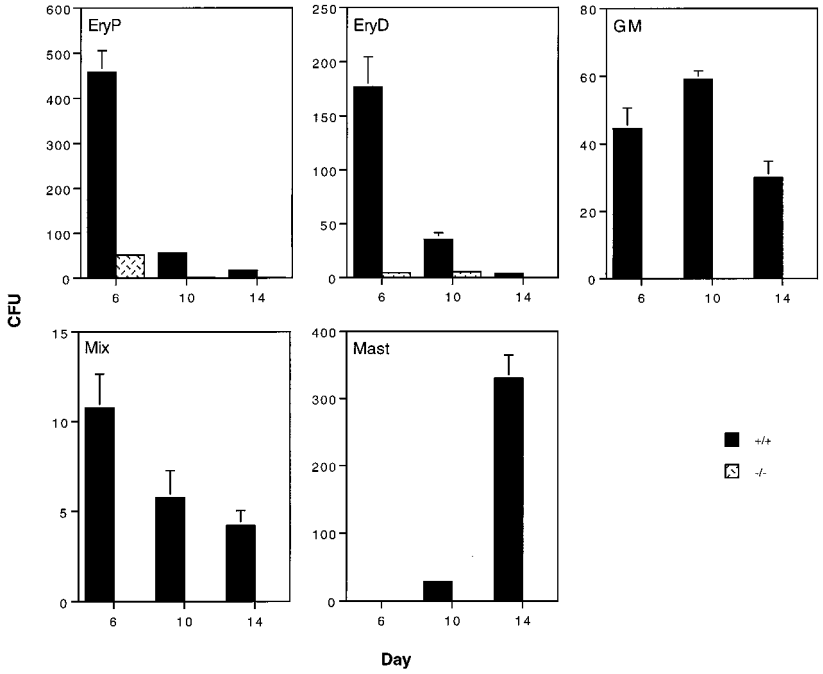


FIG. 4. The kinetics of progenitor cell development in EBs. Wild-type (+/+) and homozygous (−/−) mutant ES cell-derived EBs were harvested at the indicated times of primary culture, disrupted, and replated as single cells in the presence of different cytokines as described in the text. The colonies were counted on day 7 for $Ery^P$ and $Ery^D$ colonies and on day 12 for GM colonies, mixed erythroid-myeloid (Mix), and mast cell colonies.

to see if the mutant Shp-2 had a higher level of phosphatase activity in vitro. When identical amounts of cell lysates were immunoprecipitated with the specific anti-Shp-2 antibody and analyzed by the in vitro phosphatase assay, a higher level of dephosphorylation activity was precipitated from the homozygous mutant cells than that from wild-type cells. Since the amount of the mutant protein was only 25% of that of the wild type, this result indicates that the mutant protein has enhanced phosphatase activity in vitro (Fig. 6), in agreement with the idea that the SH2 domain might have an inhibitory role on its catalytic activity (14, 40, 41, 59).

**Deficient MAP kinase activation by SCF.** SCF serves as a growth factor for erythroid and myeloid cell differentiation both in vivo and in the in vitro ES cell differentiation assay (26, 64). c-kit, the receptor for SCF, was steadily expressed in both undifferentiated ES cells and differentiating EBs, and this expression profile was not changed in Shp-2 mutant ES cells and EBs (Fig. 5B). Apparently, the impaired hematopoiesis in mutant EBs was due not to a defect at the receptor level but rather to a disruption of cytoplasmic signaling cascades. Treatment of serum-starved wild-type ES cells with SCF (100 ng/ml) for 5, 10, and 30 min induced a transient activation of MAP kinase activity, with a maximal level of 2.5-fold at 10 min. In contrast, no significant activation was observed in homozygous Shp-2 mutant ES cells (Fig. 7). Similar results were obtained when wild-type and mutant ES cells were stimulated with epidermal growth factor.

## DISCUSSION

In this report, we provide both morphological and molecular evidence that Shp-2 plays a critical role in hematopoiesis. Significantly reduced hematopoietic activity was observed upon in vitro differentiation of homozygous Shp-2 mutant ES cells. Colony assays demonstrated that the production of both $Ery^P$ and $Ery^D$ precursors was impaired. Moreover, precursors for myeloid cells, mast cells, and mixed erythroid-myeloid cells were not detected. These in vitro findings correlate well with the defective hematopoiesis observed for mutant embryos. We previously found that Shp-2 mutants exhibit an abnormally thin and wrinkled yolk sac, with no thick-walled blood vessels, and contain only a few primitive erythrocytes (48).

Since differentiation of ES cells into blood cells in vitro closely parallels hematopoiesis in vivo (26), this in vitro system permits dissection of hematopoietic defects independent of embryonic lethality of germ line mutations, as shown previously in studies of several transcription factors, GATA-1, GATA-2, and scl (45, 47, 60, 64) and of a signaling molecule, vav (76, 77). In our experiments, we found that myeloid development was entirely blocked, whereas some erythropoiesis, albeit at a markedly reduced level, was observed. This suggests that the requirement for Shp-2 may be more stringent in the myeloid than in the erythroid lineage and that the hematopoietic defects resulting from the Shp-2 mutation are cell intrinsic. In agreement with this conclusion, we have observed significant reduction of erythroid and myeloid colony formation from human primary cord blood $CD34^+$ cells following transduction of a retrovirus vector expressing the Shp-2 SH2 domains (19, 33). It is not yet clear at what stage(s) the Shp-2 mutation affects hematopoietic cell commitment and differentiation. Since Shp-2 is widely expressed, it is likely that it functions at multiple control points. Previous biochemical data also demonstrate that Shp-2 might participate in signal relay downstream of receptors for many hematopoietic growth factors, including SCF, IL-3, Epo, and GM-CSF (56–58, 66).

A mutant Shp-2 protein with a deletion in the SH2-N domain was expressed at a lower level in heterozygous and homozygous mutant cells (25% of that of the wild type) (Fig. 1). As expected, the mutant protein retains and even has higher phosphatase activity as measured on an artificial substrate in

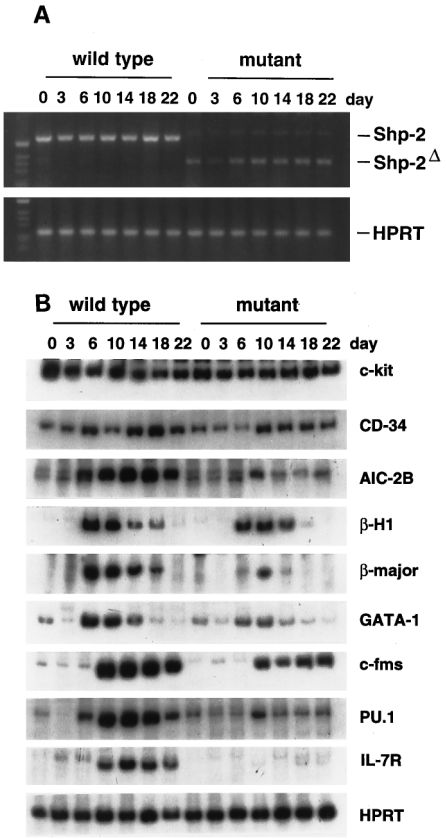


FIG. 5. Detection of gene expression by RT-PCR analysis. ES cells were seeded into semisolid methylcellulose IMDM medium. EBs were harvested at days 0, 3, 6, 10, 14, 18, and 22 of differentiation, total RNA was extracted, and a random hexamer-primed cDNA strand was synthesized by using mouse mammary leukemia virus RT. (A) Expression of *Shp-2* was examined by RT-PCR with primers as described in the legend to Fig. 1B. (B) The RT products were used as templates for PCR amplification in the presence of [α-$^{32}$P]dCTP by using gene-specific pairs of primers for c-*kit*, *CD-34*, *AIC-2B*, β-*H1*, β-*Major*, *GATA-1*, c-*fms*, *PU.1*, *IL-7R*, and *HPRT* (35, 64, 68). The relative expression level of HPRT was used as an internal control.

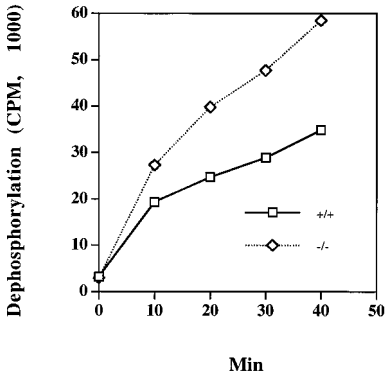


FIG. 6. Phosphatase assay. Same amounts of ES cell lysates (500 μg) of wild-type and mutant origins were immunoprecipitated by 1 μg of anti-Shp-2 antibody and subjected to in vitro phosphatase assay with $^{32}$P-labelled MBP as a substrate (20). The experiments were performed three times with reproducible results.

an in vitro assay (Fig. 6). This raises a concern of whether the defective phenotype is due to the enhanced catalytic activity or is contributed by the loss of function in the SH2 domains. Since no abnormal phenotype was observed from the heterozygous mutant animals and ES cells, this mutant protein does not appear to interfere with the normal function of the wild-type protein. Homozygous mutant mice die in utero at midgestation, with multiple defects in gastrulation and embryonic patterning (48). These include defective production of somites and the vascular endothelium, as well as perturbation of the axial mesoderm, as evidenced by abnormalities in the anterior-posterior axis, node, and notochord. It should be noted that although its phenotype was not well characterized, a Shp-2 null mutant died at the same time as our homozygous Shp-2 mutant embryo, suggesting that the mutant Shp-2 protein that we have generated is nonfunctional in cells (4, 48). Moreover, our result that Shp-2 is required for mesodermal patterning and posterior development during mouse embryogenesis is in agreement with the previous finding achieved by microinjection of catalytically inactive mutant Shp-2 mRNA into *Xenopus* embryos (55). Similarly, the phenotype of FGF-R1 knockout mouse embryos is also consistent with the results obtained from dominant-negative studies by microinjection of mutant mRNAs into *Xenopus* embryos (3, 15, 71). Therefore, while we cannot rule out the possibility that the mutant protein has some function in vivo, no available evidence would argue that the abnormal phenotypes observed in our Shp-2 mutant ES cells or embryos result from the enhanced phosphatase activity. The intact SH2 domains of Shp-2 are essential for its physiological function, and enhancement of its catalytic activity is not sufficient. Although the catalytic activity of the mutant protein is retained, the protein is biologically inert possibly because of its failure to interact with the targets or regulators via its SH2 domains. In agreement with this idea is the previous finding that a truncated protein containing the SH2 domains interfered with Shp-2 function in cells (70). Microinjection of a purified glutathione *S*-transferase–Shp-2 SH2 fusion protein into fibroblast cells inhibits DNA synthesis stimulated by epidermal growth factor, insulin, and insulin-like growth factor-I.

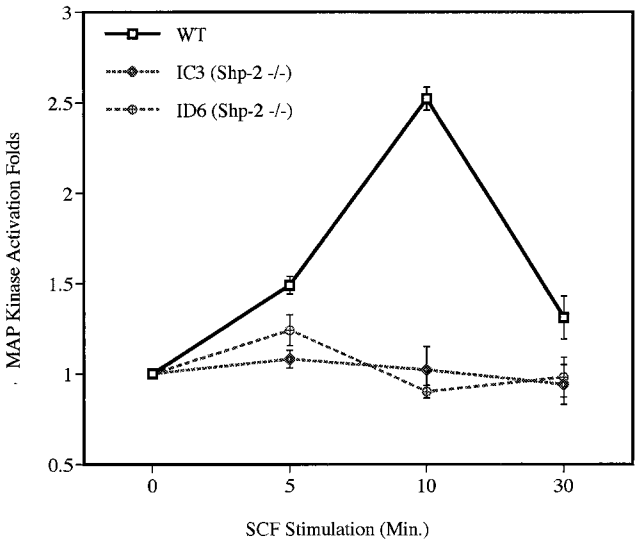


FIG. 7. MAP kinase activation by SCF. Serum-starved ES cells were treated with SCF (100 ng/ml) for 0, 5, 10, and 30 min. Cell lysates were made, and protein concentrations were measured. MAP kinases were immunoprecipitated from wild-type (WT) and mutant cell lysates by a polyclonal antibody that recognizes Erk1. The kinase activity was measured with MBP as a substrate as described in the text.

We have also observed an inhibitory effect of the Shp-2 SH2 domains on hematopoietic cell differentiation from human $CD34^+$ stem cells and TF1 cells (19, 33).

The in vitro ES cell differentiation assay has been used most extensively for the analysis of genes predominantly expressed within blood cells (45, 60, 64, 76, 77). Our results demonstrate that this approach can also be of value in dissecting cytoplasmic signaling pathways. To assess the capacity of Shp-2 mutant ES cells to differentiate into blood cells in vivo, we have attempted to generate chimeric mice by aggregating the homozygous mutant ES cells with normal eight-cell-stage embryos. We have observed that all strong chimeras die in utero, most likely due to multiple developmental defects generated in embryos with high percentages of mutant cells. Shp-2 is a ubiquitously expressed enzyme and appears to function downstream of receptors for growth factors and cytokines (20, 23, 30, 34).

In summary, we have identified Shp-2 as an indispensable positive regulator of hematopoietic development. This function stands in contrast to the negative regulatory role of Shp-1 in hematopoiesis. Although the precise molecular basis is not yet resolved, it is intriguing to note that SCF induction of MAP kinase activity is blocked in homozygous mutant ES cells (Fig. 7), thereby suggesting that blockage of SCF-induced MAP kinase might be one of the defects that is responsible for impaired hematopoiesis.

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